

3/PKTS

09/581894  
430 Rec'd PCT/PTO 16 JUN 2000

## TITLE OF THE INVENTION

C-TERMINAL REGION OF AGOUTI-RELATED TRANSCRIPT (ART) PROTEIN

## CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not applicable

## STATEMENT REGARDING FEDERALLY-SPONSORED R&amp;D

Not applicable

## 10 REFERENCE TO MICROFICHE APPENDIX

Not applicable

## FIELD OF THE INVENTION

15 The present invention is directed to polypeptides derived from the C-terminal region of the agouti-related transcript (ART) protein and to uses of such polypeptides, including use as inhibitors of the binding of melanocyte stimulating hormones to melanocortin receptors, and use to identify inhibitors of the binding of ART protein to melanocortin receptors.

## 20 BACKGROUND OF THE INVENTION

ART (agouti related transcript) was originally discovered as an mRNA that is upregulated in the hypothalamus of ob/ob and db/db mice. The ART gene has been cloned from both mice and humans and encodes a protein of 131 amino acids in mice and 132 amino acids in humans (Shutter *et al.*, 1997, Genes and Development 11:593-602). Recombinantly produced ART protein has been shown to be a functional antagonist of the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R) (Fong *et al.*, 1997, Biochem. Biophys. Res. Comm. 237:629-631; Ollman *et al.*, 1997, Science 278:135-138).

30 MC3R and MC4R belong to a class of G-protein coupled receptors known as the melanocortin receptors, since these receptors activate adenylyl cyclase in response to ligands known as melanocortins (*e.g.*, adrenocorticotrophin (ACTH) and the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -melanocyte stimulating hormones). MC3R and MC4R are neural melanocortin receptors, with MC3R being expressed in the hypothalamus and limbic system of the brain and MC4R being expressed widely in the brain. In

particular, MC4R expression has been found in a number of hypothalamic sites, including the ventromedial, lateral, dorsomedial, and paraventricular nuclei (Mountjoy *et al.*, 1994, Mol. Endocrinol. 8:1298-1308), regions which have been shown to play a role in feeding behavior (Bray, 1987, Nutr. Rev. 45:33-43). Gene targeting experiments have shown that MC4R has an important role in the control of feeding behavior and obesity. Knockout mice lacking MC4R develop an obesity syndrome characterized by hyperphagia, hyperinsulinemia, and hyperglycemia (Huszar *et al.*, 1997, Cell 88:131-141).

In view of this, there is great interest in the ART protein, which appears to be a natural regulator of MC3R and MC4R in humans. It is believed that the ART protein is likely to be a natural regulator of human obesity which functions by antagonizing either MC3R or MC4R. Accordingly, the identification of substances that inhibit the binding of ART protein to MC3R or MC4R is desirable, since such inhibitors are likely to be of value in the control of obesity. Substances that potentiate the effect of ART protein on MC3R or MC4R are also likely to be of value in the control of body weight.

#### SUMMARY OF THE INVENTION

The present invention provides novel polypeptides derived from the C-terminal region of the human and mouse ART proteins. Also provided are DNA sequences encoding the novel C-terminal polypeptides. The novel C-terminal polypeptides can be used to inhibit the binding of melanocyte stimulating hormones to melanocortin receptors. Methods of identifying inhibitors of the effect of ART protein on the binding of melanocyte stimulating hormones to melanocortin receptors are also provided.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the quantitation of c-ART-b from COS-7 cells through the use of an ELISA using an antibody that recognizes the myc epitope in c-ART-b. Shown is the standard curve generated using known amounts of myc peptide. For the c-ART-b preparation made from COS-7 cells, a 10x dilution of the sample gave an absorbance of 0.079, corresponding to 10 nM in the above standard curve. Therefore, the c-ART-b preparation had a concentration of 100 nM.

Figure 2 shows the binding affinity of c-ART-b for the human MC3R. Shown is the inhibition of  $^{125}\text{I}$ -[Tyr<sup>2</sup>][Nle<sup>4</sup>, D-Phe<sup>7</sup>]  $\alpha$ -melanocyte stimulating hormone ( $^{125}\text{I}$ -NDP- $\alpha$ -MSH) binding to the human MC3R by c-ART-b.

Figure 3 shows the binding affinity of c-ART-b for the human MC4R. Shown is the inhibition of  $^{125}\text{I}$ -NDP- $\alpha$ -MSH binding to the human MC4R by c-ART-b.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides C-terminal polypeptides derived from the agouti-related transcript (ART) protein and DNA sequences encoding those polypeptides. The C-terminal polypeptides from the ART protein are referred to herein as "ART polypeptides". In certain embodiments, the ART polypeptides are present in a contiguous polypeptide sequence, *i.e.*, a fusion protein, that incorporates, generally at the C-terminus of the fusion protein, one or more amino acid sequences not derived from the ART protein. Such non-ART protein sequences can be, *e.g.*, "tags", such as a protein kinase A site (for easier radioisotope labeling) or an antigenic sequence (*e.g.*, a myc epitope) for ELISA quantitation. Other tags are known in the art and ART polypeptides incorporating such other tags are included in the present invention. In other embodiments, the ART polypeptides are present in a fusion protein with another protein that gives rise to an easily detectable signal, *e.g.*, alkaline phosphatase (ART-AP) or luciferase (ART-luc). Fusion proteins such as ART-AP or ART-luc are useful in binding assays since their presence and/or concentration can be detected without the use of radioactivity.

In particular, the present invention includes the following ART polypeptides:

c-ART-a: This polypeptide contains, from N to C terminus: (1) a yeast signal sequence peptide; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4) a myc epitope; (5) a protein kinase A (PKA) site; and (6) a hexahistidine tag. The amino acid sequence of c-ART-a is:

MNIFYIFLFLLSFVQGLEHTRRGSLVKRSSLQDREPRS

1

SRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLG

2

5 TAMNPCSRTLLVPRGSEQKLISEEDLNLRRASLGHHHHHH

3

4

5

6

(SEQ.ID.NO.:1)

c-ART-b: This polypeptide contains, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4) a myc epitope; and (5) a hexahistidine tag. The amino acid sequence of c-ART-b is:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRCVRL

15

1

HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCS

2

RTLLVPRGSGSELGTKLGPEQKLISEEDLNSAVDHHHHHH

3

4

5

20 (SEQ.ID.NO.:2)

c-ART-c: This polypeptide contains, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4) a PKA site; (5) a myc epitope; and (6) a hexahistidine tag. The amino acid sequence of c-ART-c is:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRCVRL

1

30 HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT

2

LVPRGSGSLRRASLGKLEQKLISEEDLNSAVDHHHHHH

3

4

5

6

(SEQ.ID.NO.:3)

ART-AP: This polypeptide contains, from N to C terminus: (1) amino acids 1-132 of the human ART protein; (2) a thrombin site; (3) the alkaline phosphatase protein; (4) a myc epitope; and (5) a hexahistidine tag. The amino acid sequence of ART-AP is:

5

MLTAALLSCALLLALPATRGAQMGLAPMEGIRRPDQALLP

ELPGLGLRAPLKKTNAEQAEEDLLQEAQALAEVLDLQDRE

1

10 PRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR

KLGTAMNPCSRTLVPRGSGSIIPVEEENPDFWNRQAAEAL

2

GAAKKLQPAQTAAKNLIIFLGDMGVSTVTAARILKGQKK

15

DKLGPETFLAMDRFPYVALSKTYSVDKHVPDSGATATAYL

CGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAG

20 KSVGVTTRVQHASPAGAYAHTVNERNWYSDADVPASA

RQEGCQDIATQLISNMDIDVILGGGRKYMFPMTGTPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTELM

25

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSLMEMTE

3

AALRLLSRNPRGFFLFVEGGRIDHGHESRAYRALTETIM

30 FDDAIERAGOLTSEEDTLSLVTADHSHVFSFGGYPLRGSS

IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFI AHVMAFAACLEPYTACDLAPSAGTTDAAHPG

KLGP E QKLISEEDLNSAVDHHHHHH (SEQ.ID.NO.:4)

5

4

5

ART-luc: This polypeptide contains, from N to C terminus: (1) amino acids 1-132 of the human ART protein; (2) a thrombin site; (3) the luciferase protein; (4) a myc epitope; and (5) a hexahistidine tag. The amino acid sequence of ART-luc is:

10

MLTAALLSCALLALPATRGAQMGLAPMEGIRRPDQALLP

ELPGLGLRAPLKKTNAEQAEEDLLQEAQALAEVLDLQDRE

15

1

PRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR

KLGTAMNPCSRTL VPRGSGSMSIENNILIGPPPYPLEEG

2

20

TAGEQLHRAISRYAAVPGTLAYTDVHTELEVITYKEFLDVT

CRLAEAMKNYGLGLQHTISVCSENCVQFFMPICAALYVG

VATAPTNDIYNERELYNLSISQPTVVFTSRNSLQKILGVQ

25

SRLPIIKKIIILDGKKDYLGYSMQSFMKEHVPANFNVSA

FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH

30

CRDPVFGNQIIPDTTILCAVPFHHAFGTFTNLGYLICGFH

VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL

3

VDKYDLSNLHEIASGGAPLSKEISEIAAKRFLPGIRQGYG

LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK

LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG

5

DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLQH

PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY

10

VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK

AQKGKSKSAKLGPEQKLISEEDLNSAVDHHHHHH (SEQ.ID.NO.:5)

4

5

15

The present invention also includes an ART polypeptide containing amino acids 1-26 and 76-132 of the human ART protein, having the following polypeptide sequence:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRCVRL

20

HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT  
(SEQ.ID.NO.:6)

25

The present invention also includes an ART polypeptide containing amino acids 76-132 of the human ART protein, having the following polypeptide sequence:

LQDREPRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMN  
PCSRT (SEQ.ID.NO.:7)

30

The present invention also includes an ART polypeptide containing amino acids 1-26 and 75-131 of the mouse ART protein, having the following polypeptide sequence:

MLTAMLLSCVLLLALPPTLGVQMGPVAPQNRRESRSPRRCVRL  
HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLG TAMNLC SRT  
(SEQ.ID.NO.:8)

5           The present invention also includes an ART polypeptide containing amino acids 75-131 of the mouse ART protein, having the following polypeptide sequence:

10    PQNRESRSPRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMN  
      LCSRT (SEQ.ID.NO.:9)

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) a yeast signal sequence peptide; (2) amino acids 75-131 of the mouse ART protein; (3) a thrombin site; (4) a myc epitope; (5) a PKA site; and (6) a hexahistidine tag. The amino acid sequence of this ART polypeptide is:

MNIFYIFLFLLSFVQGLEHThRRGSLVKRSSPQNRESRSPRRCVRL  
 1  
 20 HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGtAMNLCsRT  
 2  
LVPRGSEQKLISEEDLNLRRASLGHHHHHH (SEQ.ID.NO.:10)  
 3 4 5 6

25                   The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-26 of the mouse ART protein; (2) amino acids 75-131 of the mouse ART protein; (3) a thrombin site; (4) a myc epitope; and (5) a hexahistidine tag. The amino acid sequence of this ART polypeptide is:

30

MLTAMLLSCVLLLALPPTLGVQMGVAPQNRESRSPRRCVRL

1

HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLG TAMNLC SRT

2



LVPRGSEQKLISEEDLNLRRASLSHHHHHH (SEQ.ID.NO.:11)

3 4 5 6

5 The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-26 of the mouse ART protein; (2) amino acids 75-131 of the mouse ART protein; (3) a thrombin site; (4) PKA site; (5) a myc epitope; and (6) a hexahistidine tag. The amino acid sequence of this ART polypeptide is:

10 MLTAMLLSCVLLLALPPTLGVQMGMVAPQNRRESRSPRRCVRL

1

HESCLGQQVPCCDPCATCYCRFFNAFCYCRKLG TAMNLC SRT

2

LVPRGSGSLRRASLGKLEQKLISEEDLNHHHHHH

15 3 4 5 6

(SEQ.ID.NO.:12)

20 The present invention also includes an ART polypeptide, having the following sequence, from N to C terminus: (1) amino acids 1-131 of the mouse ART protein; and (2) the alkaline phosphatase protein. The amino acid sequence of this polypeptide is:

MLTAMLLSCVLLLALPPTLGVQMGMVAPLKGIRRPDQAL

25 FP EFPGLSLNGLKKTNADRAEEVLLQKAEALAEVLDP

1

QNRRESRSPRRCVRLHESCLGQQVPCCDPCATCYCRFFN

AFCYCRKLG TAMNLC SRTIIPVEEENPDFWNRQAAEAL

30

GAAKKLQPAQTA AKNLIIFLGDMGVSTVTAARILKGQKK

DKLGPETFLAMDRFPYVALSKTYSVDKHVPDSGATATAYL

CGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAG

KSVGVTTRVQHASPAGAYAHTVNRNWYSDADVPASA

5 RQEGCQDIATQLISNMDIDVILGGGRKYMFPMTGTPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTELM

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSTLMEMTE

10

2

AALRLLSRNPRGFFLFVEGGRIDHGHESRAYRALTETIM

FDDAIERAGQLTSEEDTSLVTADHSHVFSFGGYPLRGSS

15 IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

20

(SEQ.ID.NO.:13)

The present invention also includes an ART polypeptide, having the following sequence, from N to C terminus: (1) amino acids 1-131 of the mouse ART protein; and (2) the luciferase protein. The amino acid sequence of this polypeptide is:

25

MLTAMLLSCVLLLALPPTLGVQMGVAPLKGIRRPDQALFP

EFPGLSLNLKKTNADRAEEVLLQKAEALAEVLDPQNRES

30

1

RSPRRCVRLHESCLGQOVPCCDPCATCYCRFFNAFCYCRK

LGTAMNLCSTRTMSIENNILIGPPPYPLEEGTAGEQLHR

AISRYAAVPGTLAYTDVHTELEVITYKEFLDVTCLAEAE  
 MKNYGLGLQHTISVCSENCVQFFMPICAAALYVGVATAP  
 5 TNDIYNERELYNSLSISQPTVVFTSRNSLQKILGVQSR  
 LPIKKIILDGKKDYLGYSMSFMKEHVPANFNVSA  
 FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH  
 10 CRDPVFGNQIIPDTTILCAVPFHHAFGTFTNLGYLICGFH  
 VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL  
 2  
 15 VDKYDLSNLHEIASGGAPLSKEISEIAAKRFLPGIRQGYG  
 LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK  
 LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG  
 20 DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLQH  
 PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY  
 25 VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK  
 AQKGKSKSKAKL (SEQ.ID.NO.:14)

30 The present invention also includes an ART polypeptide, having the  
 following sequence, from N to C terminus: (1) amino acids 1-26 of the human ART  
 protein; (2) amino acids 76-132 of the human ART protein; (3) a thrombin site; (4)  
 the alkaline phosphatase protein; (5) a myc epitope; and (6) a hexahistidine tag. The  
 amino acid sequence of this polypeptide is:

MLTAALLSCALLLALPATRGAQMGLALQDREPRSSRRCV

1

RLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTA

2

5 MNPCSRTLVPRGSGSIIPVEEENPDFWNRQAAEAL

3

GAAKKLQPAQTAAKNLIIFLGDMGVSTVTAARILKGQKK

DKLGPETFLAMDRFPYVALSKTYSVDKHVPDSGATATAYL

10

CGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAG

KSVGVTTRVQHASPAGAYAHTVNRNWYSDADVPA

15 RQEGCQDIATQLISNMDIDVILGGGRKYMFPMTGPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTLM

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSLMEMTE

20

4

AALRLLSRNPRGFFLFVEGGRIDHGHHSRAYRALTETIM

FDDAIERAGQLTSEEDTLSTADHSHVFSFGGYPLRGSS

25 IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

30

KLGPEQKLISEEDLNSAVDHHHHHH (SEQ.ID.NO.:15)

5

6

5

10

15

20

25

30

DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLQH

PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY

VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK

5    AQKGKSKSKAKLGPEQKLISEEDLNSAVDHHHHHH (SEQ.ID.NO.:16)

5

6

10                    The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-132 of the human ART protein; and (2) the alkaline phosphatase protein. The amino acid sequence of this polypeptide is:

MLTAALLSCALLLALPATRGAQMGLAPMEGIRRPDQALLP

15    ELPGLGLRAPLKKTNAEQAEEDLLQEAQALAEVLDLQDRE

1

PRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR

KLGTAMNPCSRTIIPVEEENPDFWNRQAAEALGAACKKLQPA

20

QTAACKNLIIFLDGDMGVSTVTAARILKGQKKDKLGPETF

LAMDRFPYVALSKTYSVDKHVPDSGATATAYLCGVKGN

25    FQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAGKSVG

VVTTTRVQHASPAGAYAHTVNRNWYSADVPASARQ

EGCQDIATQLISNMDIDVILGGGRKYMFPMTGTPDPEY

30

PDDYSQGGTRLGDKNLVQEWLAKHQGARYVWNRTELM

QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPSLMEMTE

2

AALRLLSRNPRGFFLFVEGGRIDHGHESRAYRALTETIM

FDDAIERAGQLTSEEDTLSTADHSHVFSFGGYPLRGSS

5 IFGLAPGKARDRKAYTVLLYGNGPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

VQEQTFAIAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

10

(SEQ.ID.NO.:17)

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-132 of the human ART protein; and (2) the luciferase protein. The amino acid sequence of this polypeptide is:

15

MLTAALLSCALLLALPATRGAQMGLAPMEGIRRPDQALLP

20

ELPGLGLRAPLKKTNAEQAEEDLLQEAQALAEVLDLQDRE

1

PRSSRRCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR

KLGTAMNPCSRTMSIENNILIGPPPYYPLEEGTAGEQLH

25

RAISRYAAVPGTLAYTDVHTELEVITYKEFLDVTCRLAE

AMKNYGLGLQHTISVCSENCVQFFMPICAALYVGVAT

30

APTNDIYNERELYNSLSISQPTVVFTSRNSLQKILGVQ

SRLPIIKKIILDGKKDYLGYSQSMQSFMEHVPANFNVSA

FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH

CRDPVFGNQIIPDTTILCAVPFHHAFGTFTNLGYLICGFH

VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL

5

2

VDKYDLSNLHEIASGGAPLSKEISEIAAKRFKLPGIRQGYG

LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK

10

LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG

DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLQH

PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY

15

VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK

AQKGKSKSKAKL (SEQ.ID.NO.:18)

20

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) the alkaline phosphatase protein; (3) amino acids 27-132 of the human ART protein. The amino acid sequence is:

25 MLTAALLSCALLLALPATRGAQMGLAIIPVEEENPDFWNRQAAEAL

1

GAAKKLQPAQTAAKNLIIFLGDMGVSTVTAARILKGQKK

DKLGPETFLAMDRFPYVALSKTYSVDKHVPD SGATATAYL

30

CGVKGNFQTIGLSAAARFNQCNTTRGNEVISVMNRAKKAG

KSVGVTTRVQHASPAGAYAHTVNRNWYSDADVPASA



RQEGCQDIATQLISNMDIDVILGGGRKYMFPMPGTPDPEY

PDDYSQGGTRLDGKNLVQEWLAKHQGARYVWNRTELM

5 QASLDPSVTHLMGLFEPGDMKYEIHRDSTLDPslMEMTE

2

AALRLLSRNPRGFFLFVEGGRIDHGHESRAYRALTETIM

FDDAIERAGQLTSEEDTLslVTADHSHVFSFGGYPLRGSS

10

IFGLAPGKARDRKAYTVLLYGNPGYVLKDGARPDVTES

ESGSPEYRQQSAVPLDGETHAGEDVAVFARGPQAHLVHG

15 VQEQTfIAHVMAFAACLEPYTACDLAPSAGTTDAAHPG

PMEGIRRPDQALLPELPGLGLRAPLKKTNAEQAEEDLLQE

3

AQALAEVLDLQDREPRSSRRCVRLHESCLGQQVPCCDPC

20

ATCYCRFFNAFCYCRKLGTAMNPCSRT (SEQ.ID.NO.:19)

The present invention also includes an ART polypeptide having the following sequence, from N to C terminus: (1) amino acids 1-26 of the human ART protein; (2) the luciferase protein; (3) amino acids 27-132 of the human ART protein. The amino acid sequence is:

MLTAALLSCALLLALPATRGAQMGLAMSIENNILIGPPPYYPLEEG

1

30 TAGEQLHRAISRYAAVPGTLAYTDVHTELEVITYKEFLDVT

CRLAEAMKNYGLGLQHTISVCSENCVQFFMPICAALYVG

VATAPTNDIYNERELYNSLSISQPTVVFTSRNSLQKILGVQ

SRLPIIKKIILDGKKDYLGYSMQSFMKEHVPANFNVSA

FKPLSFDLDRVACIMNSSGSTGLPKGVPISHRNTIYRFSH

5

CRDPVFGNQIIPDTTILCAVPFHHAFTNLGYLICGFH

VVLMYRFNEHLFLQTLQDYKCQSALLVPTVLAFLAKNPL

2

10

VDKYDLSNLHEIASGGAPLSKEISEIAAKRFKLPGIRQGYG

LTETTCAIVITAEGEFKLGAVGKVVPFYSLKVLDLNTGKK

LGPNERGEICFKGPMIMKGYINNPEATRELIDEEGWIHSG

15

DIGYFDEDGHVYIVDRLKSLIKYKGYQVPPAELEALLQH

PFIEDAGVAGVPDEVAGDLPGAVVVLKEGKSITEKEIQDY

20

VAGQVTSSKKLRGGVEFVKEVPKGFTGKIDTRKIKEILIK

AQKGKSKSKAKLPMEGIRRPDQALLPELPGLGLRAPLKK

TNAEQAEEDLLQEAQALAEVLDLQDREPRSSRRCVRLHE

25

3

SCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT

(SEQ.ID.NO.:20)

30

The ART polypeptides of the present invention can be in a form that is substantially free from other polypeptides. "Substantially free from other polypeptides" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other proteins. Thus, an ART polypeptide preparation that is substantially free from other polypeptides will contain, as a percent of its total

polypeptides, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-ART polypeptides. Whether a given ART polypeptide preparation is substantially free from other polypeptides can be determined by such conventional techniques as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate staining methods, *e.g.*, silver staining.

It is possible to modify many of the amino acids of the ART polypeptides of the present invention and still retain substantially the same biological activity as possessed by the unmodified ART polypeptide. A modified ART polypeptide has "substantially the same biological activity" as an unmodified ART polypeptide if the modified polypeptide has an IC<sub>50</sub> value for the inhibition of <sup>125</sup>I-labeled NDP- $\alpha$ -MSH binding to MC3R or MC4R that is no more than 5-fold greater than the IC<sub>50</sub> value of the unmodified ART polypeptide for the inhibition of <sup>125</sup>I-labeled NDP- $\alpha$ -MSH binding to MC3R or MC4R.

Thus the present invention includes modified ART polypeptides which have amino acid deletions, additions, or substitutions but that still retain substantially the same biological activity as the unmodified ART polypeptide from which they are derived. It is generally accepted that single amino acid substitutions at non-critical positions do not usually alter the biological activity of a protein or polypeptide (see, *e.g.*, Molecular Biology of the Gene, Watson *et al.*, 1987, Fourth Ed., The Benjamin/Cummings Publishing Co., Inc., page 226; and Cunningham & Wells, 1989, Science 244:1081-1085). Accordingly, the present invention includes modified polypeptides where one amino acid substitution has been made in SEQ.ID.NOs.:1-20 wherein the modified polypeptides still retain substantially the same biological activity as the unmodified ART polypeptides. The present invention also includes modified polypeptides where two amino acid substitutions have been made in SEQ.ID.NOs.:1-20 wherein the polypeptides still retain substantially the same biological activity as the unmodified ART polypeptides. More generally, the present invention includes modified polypeptides where amino acid substitutions have been made in regions of the polypeptides that are not critical, *i.e.*, in regions where modifications result in a polypeptide with substantially the same biological activity as the unmodified polypeptide.

In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. A "conservative amino

acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (*e.g.*, arginine for lysine; glutamic acid for aspartic acid).

The present invention also includes DNA sequences encoding polypeptides having the amino acid sequences of SEQ.ID.NOs.:1-20, with the proviso that, In the case of the DNA sequences encoding SEQ.ID.NOs.:6-9, the DNA sequences do not encode any contiguous stretch of amino acids from the ART protein other than SEQ.ID.NOs.:6-9.

The DNA sequences of the present invention can be in a form that is substantially free from other nucleic acids. "Substantially free from other nucleic acids" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other nucleic acids. Thus, a preparation of DNA sequences encoding an ART polypeptide that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acids, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of nucleic acids other than the DNA sequences encoding ART polypeptides. Whether a given preparation of DNA sequences encoding an ART polypeptide is substantially free from other nucleic acids can be determined by such conventional techniques as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*, ethidium bromide staining.

The DNA sequences of the present invention encoding ART polypeptides can be linked with other DNA sequences, *e.g.*, DNA sequences to which DNA sequences encoding the ART protein are not naturally linked, to form "recombinant DNA molecules" encoding ART polypeptides. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms, or that confer antibiotic resistance. The DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, or yeast artificial chromosomes.

Included in the present invention are DNA sequences that hybridize to the DNA sequences encoding ART polypeptides under stringent conditions. By way

of example and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10<sup>6</sup> cpm of <sup>32</sup>P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would include either a hybridization carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, *e.g.*, Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding ART polypeptides. Such recombinant host cells can be cultured under suitable conditions to produce ART polypeptides. An expression vector containing DNA encoding ART polypeptides can be used for expression of ART polypeptides in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cell lines derived from mammalian species which are suitable for recombinant expression of ART polypeptides and which are commercially available, include but are not limited to, L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171).

A variety of mammalian expression vectors can be used to express ART polypeptides in mammalian cells. Commercially available mammalian expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNA1 and pcDNA1amp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), and pSV2-dhfr (ATCC 37146). Following expression in recombinant cells, ART polypeptides can be purified by conventional techniques to a level that is substantially free from other proteins.

The present invention includes a method of determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor. Such substances are likely to be useful in the control of body weight. The method takes advantage of the fact that ART polypeptides inhibit the binding of melanocyte stimulating hormones to melanocortin receptors by themselves binding to the receptor. Thus, a substance that antagonizes the inhibitory effect of ART polypeptides on the binding of melanocyte stimulating hormones to melanocortin receptors is likely to act by inhibiting the binding of the ART polypeptide itself to the melanocortin receptor. The method comprises:

- (a) providing cells expressing the melanocortin receptor;
- (b) exposing the cells to a chosen concentration of the melanocyte stimulating hormone in the absence of the ART polypeptide and in the absence of the substance and measuring the amount of melanocyte stimulating hormone binding to the cells to obtain a first value for melanocyte stimulating hormone binding;
- (c) exposing the cells to the chosen concentration of melanocyte stimulating hormone in the presence of a chosen concentration of the ART polypeptide and in the absence of the substance and measuring the amount of melanocyte stimulating hormone binding to obtain a second value for melanocyte stimulating hormone binding where the second value for melanocyte stimulating hormone binding indicates that less melanocyte stimulating hormone binding has occurred as compared to the first value for melanocyte stimulating hormone binding;
- (d) exposing the cells to the chosen concentration of melanocyte stimulating hormone in the presence of the chosen concentration of ART polypeptide and in the presence of the substance and measuring the amount of melanocyte

stimulating hormone binding to obtain a third value for melanocyte stimulating hormone binding;

where, if the third value for melanocyte stimulating hormone binding is greater than the second value, then the substance is an inhibitor of the binding of the ART polypeptide to the melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method known in the art for the introduction of the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells, and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 cells (ATCC CRL 1573), Raji cells (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-

C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the melanocyte stimulating hormone is selected from the group consisting of:  $\alpha$ -melanocyte stimulating hormone,  $\beta$ -melanocyte stimulating hormone, and  $\gamma$ -melanocyte stimulating hormone.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs. 1-19 and 20.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions under which the method is practiced are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments of the above-described method, the chosen concentration of the melanocyte stimulating hormone is from 0.05 nM to 2.0 nM, preferably from 0.1 nM to 1.0 nM, and more preferably from 0.2 nM to 0.5 nM.

In particular embodiments of the above-described method, the chosen concentration of the ART polypeptide is from  $10^{-12}$  M to  $10^{-7}$  M.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the melanocyte stimulating hormone is labeled, *e.g.*, enzymatically, radioactively, or the like, and the amount of binding of the melanocyte stimulating hormone to the melanocortin receptor is measured by determining the amount of label bound to the cells containing the melanocortin receptor.

Steps (b), (c), and (d) of the above-described method can be modified in that, rather than exposing intact cells to the melanocyte stimulating hormone, the ART polypeptide, or the substance, membranes can be prepared from the cells and the membranes can be exposed to the melanocyte stimulating hormone, the ART polypeptide, or the substance. Such a modification utilizing membranes rather than intact cells in methods similar to that described above, although directed to the binding interactions of other ligands and receptors, is well known in the art and is described in, *e.g.*, Hess *et al*, 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding the melanocortin receptor can be prepared as, *e.g.*, by *in vitro* transcription



using a plasmid containing nucleotide sequences encoding the melanocortin receptor under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of the melanocortin receptor in the oocytes. These oocytes then take the place of the cells in the above described method.

Once a substance has been identified as an inhibitor of the binding of the ART polypeptide to the melanocortin receptor, that substance can be tested to determine whether it is also an agonist of the melanocortin receptor. Such testing would involve exposing cells that express the melanocortin receptor to the substance, in the absence of the melanocyte stimulating hormone and the ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of the effect of ART protein on MC3R or MC4R can be identified that has no, or little, MC3R or MC4R agonist activity, but that relieves the inhibition of MC3R or MC4R receptor activity produced by ART protein. In a similar manner, it can be determined whether the substance is an antagonist of the melanocortin receptor.

The present invention also includes a method for determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor where the method comprises:

- (a) providing cells expressing a melanocortin receptor;
  - (b) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would bind to the melanocortin receptor;
  - (c) measuring the amount of binding of the ART polypeptide to the melanocortin receptor in the presence and in the absence of the substance;
- where a decrease in the amount of binding of the ART polypeptide to the melanocortin receptor in the presence as compared to the absence of the substance indicates that the substance is an inhibitor of the binding of the ART polypeptide to the melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include

any method known in the art for the introduction of the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and  
 5 electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

10 The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells,  
 15 and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression  
 20 of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1  
 25 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.1-19 and 20. In particular embodiments of the above-described method, the ART polypeptide is used  
 30 in a concentration of from  $10^{-12}$  M to  $10^{-7}$  M.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions

such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the ART polypeptide is labeled, *e.g.*, enzymatically, radioactively, or the like, and the amount of binding of the ART polypeptide to the melanocortin receptor is measured by determining the amount of label bound to the melanocortin receptor. The ART polypeptide will either be radioactively labeled by <sup>32</sup>P, <sup>33</sup>P, or <sup>125</sup>I (*e.g.*, for c-ART-a or c-ART-c), or non-radioactively labeled (*e.g.*, ART-AP, ART-luc, c-ART-AP or c-ART-luc). In the case of these latter ART polypeptides, the ART polypeptides can be detected by detecting the enzymatic activity of the alkaline phosphatase or luciferase moieties of the polypeptides.

Step (b) of the above-described method can be modified in that, rather than exposing the cells to an ART polypeptide in the presence and in the absence of the substance, membranes can be prepared from the cells and the membranes can be exposed to an ART polypeptide in the presence and in the absence of the substance. Such a modification utilizing membranes rather than cells in methods similar to that described above, although directed to the binding interactions of other ligands and receptors, is well known in the art and is described in, *e.g.*, Hess *et al*, 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding a melanocortin receptor can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing nucleotide sequences encoding a melanocortin receptor under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of the melanocortin receptor in the oocytes. These oocytes then take the place of the cells in the above described method.

Once a substance has been identified as an inhibitor of ART binding to a melanocortin receptor, that substance can be tested to determine whether it is also an agonist of the melanocortin receptor. Such testing would involve exposing cells that express the melanocortin receptor to the substance, in the absence of ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of ART protein binding to MC3R or MC4R may be identified that has no, or little, MC3R or MC4R agonist activity, but

that relieves the inhibition of MC3R or MC4R receptor activity produced by ART protein.

The present invention also includes a method for determining whether a substance is an allosteric enhancer of the binding of an ART polypeptide to a melanocortin receptor where the method comprises:

- (a) providing cells expressing a melanocortin receptor;
- (b) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would bind to the melanocortin receptor;
- (c) measuring the amount of binding of the ART polypeptide to the melanocortin receptor in the presence and in the absence of the substance;

where an increase in the amount of binding of the ART polypeptide to the melanocortin receptor in the presence as compared to the absence of the substance indicates that the substance is an allosteric enhancer of the binding of the ART polypeptide to the melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method known in the art for the introduction of the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells,

and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.1-19 and 20. In particular embodiments of the above-described method, the ART polypeptide is used in a concentration of from  $10^{-12}$  M to  $10^{-7}$  M.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the ART polypeptide is labeled, *e.g.*, enzymatically, radioactively, or the like, and the amount of binding of the ART polypeptide to the melanocortin receptor is measured by determining the amount of label bound to the melanocortin receptor.

Step (b) of the above-described method can be modified in that, rather than exposing the cells to an ART polypeptide in the presence and in the absence of the substance, membranes can be prepared from the cells and the membranes can be exposed to an ART polypeptide in the presence and in the absence of the substance. Such a modification utilizing membranes rather than cells in methods similar to that described above, although directed to the binding interactions of other ligands and receptors, is well known in the art and is described in, *e.g.*, Hess *et al*, 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding a melanocortin receptor can be prepared as, *e.g.*, by *in vitro* transcription using a plasmid containing nucleotide sequences encoding a melanocortin receptor under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of the melanocortin receptor in the oocytes. These oocytes then take the place of the cells in the above described method.

Melanocortin receptors are G-protein coupled receptors that stimulate G<sub>s</sub>, leading to the production of cAMP (Cone *et al.*, 1996, Recent Prog. Hormone Res. 51:287-318). Thus, the ART polypeptides of the present invention can be used in a method for determining whether a substance is a functional inhibitor of the antagonistic effect of an ART polypeptide on a melanocortin receptor where the method comprises:

- (a) providing cells expressing a melanocortin receptor;
- (b) exposing the cells to a melanocyte stimulating hormone, thereby activating the melanocortin receptor and leading to the production of cAMP mediated by the melanocortin receptor;
- (c) exposing the cells to an ART polypeptide in the presence and in the absence of the substance under conditions such that if the substance were not present, the ART polypeptide would inhibit the production of cAMP mediated by the melanocortin receptor;
- (d) measuring the amount of cAMP produced the presence and in the absence of the substance;

where an increase in the amount of cAMP produced in the presence as compared to the absence of the substance indicates that the substance is a functional inhibitor of the antagonistic effect of the ART polypeptide on the melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method known in the art for the introduction of the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection

includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

5 In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

10 The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells, and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are  
15 selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573),  
20 Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C1271 (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

25 In a particular embodiment, the melanocyte stimulating hormone is selected from the group consisting of:  $\alpha$ -melanocyte stimulating hormone,  $\beta$ -melanocyte stimulating hormone, and  $\gamma$ -melanocyte stimulating hormone.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of:  
30 SEQ.ID.NOs.1-19 and 20.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions

such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

Once a substance has been identified as a functional inhibitor of the antagonistic effect of an ART polypeptide on a melanocortin receptor, that substance  
 5 can be tested to determine whether it is also an agonist of the melanocortin receptor. Such testing would involve exposing cells that express the melanocortin receptor to the substance, in the absence of ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of ART protein binding to MC3R or MC4R may be developed that has  
 10 no, or little, MC3R or MC4R agonist activity, but that relieves the inhibition of MC3R or MC4R receptor activity produced by ART protein. In a similar manner, it can be determined whether the substance is an antagonist of the melanocortin receptor.

The ART polypeptides of the present invention can also be used in a  
 15 method of determining whether a substance is an inhibitor of the effect of an ART polypeptide that makes use of an assay utilizing a *Xenopus* melanophore cell line (see, e.g., Quillan *et al.*, 1995, Proc. Natl. Acad. Sci. USA 92:2894; Potenza & Lerner, 1992, Pigment Cell Res. 5:372; Ollman *et al.*, 1997, Science 278:135-138). Such a method comprises:

- 20 (a) providing a *Xenopus* melanophore cell line;
- (b) exposing the *Xenopus* melanophore cell line to a chosen concentration of a melanocyte stimulating hormone in the absence of the ART polypeptide and in the absence of the substance and measuring the amount of pigment dispersion to obtain a first value for pigment dispersion;
- 25 (c) exposing the *Xenopus* melanophore cell line to the chosen concentration of  $\alpha$ -melanocyte stimulating hormone in the presence of the ART polypeptide and in the absence of the substance and measuring the amount of pigment dispersion to obtain a second value for pigment dispersion where the second value for pigment dispersion indicates that less pigment has been dispersed as compared to the  
 30 first value for pigment dispersion;
- (d) exposing the *Xenopus* melanophore cell line to the chosen concentration of  $\alpha$ -melanocyte stimulating hormone in the presence of the ART polypeptide and in the presence of the substance and measuring the amount of pigment dispersion to obtain a third value for pigment dispersion;



where if the third value for pigment dispersion indicates that more pigment has been dispersed as compared with the second value, then the substance is an inhibitor of the effect of the ART polypeptide.

5 In a particular embodiment, the melanocyte stimulating hormone is selected from the group consisting of:  $\alpha$ -melanocyte stimulating hormone,  $\beta$ -melanocyte stimulating hormone, and  $\gamma$ -melanocyte stimulating hormone.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of:  
SEQ.ID.NOs.1-19 and 20.

10 In particular embodiments of the above-described method, the conditions are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

15 Once a substance has been identified as an inhibitor of the effect of an ART polypeptide, that substance can be tested to determine whether it is also an agonist of the *Xenopus* melanocortin receptor. Such testing would involve exposing melanophore cells that express the *Xenopus* melanocortin receptor to the substance, in the absence of ART protein or ART polypeptides, and determining whether the  
20 melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of ART protein binding to the *Xenopus* melanocortin receptor can be identified that may be used as a lead to develop ART binding inhibitors for human MC3R or MC4R that have no, or little, MC3R or MC4R agonist activity, but that relieve the inhibition of MC3R or MC4R receptor activity produced by ART protein. In a similar manner,  
25 it can be determined whether the substance is an antagonist of the melanocortin receptor.

The present invention includes a method of determining whether a substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor comprising:

- 30 (a) providing cells expressing the melanocortin receptor;  
(b) exposing the cells to a chosen concentration of the melanocyte stimulating hormone and a chosen concentration of the ART polypeptide in the presence and in the absence of the substance and measuring the amount of melanocyte

stimulating hormone binding to the cells in the presence and in the absence of the substance;

where an increase in the amount of melanocyte stimulating hormone binding in the presence of the substance indicates that the substance is an inhibitor of the binding of an ART polypeptide to a melanocortin receptor.

In a particular embodiment, the cells expressing the melanocortin receptor are cells that naturally express the melanocortin receptor. In another embodiment, the cells expressing the melanocortin receptor do not naturally express the melanocortin receptor but have been transfected with an expression vector that directs the expression of the melanocortin receptor. Transfection is meant to include any method known in the art for the introduction of the expression vector directing the expression of the melanocortin receptor into the cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing the melanocortin receptor, and electroporation.

In a particular embodiment, the melanocortin receptor is selected from the group consisting of: the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R). In a particular embodiment of the above-described method, the melanocortin receptor is not a *Xenopus* melanocortin receptor.

The cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor can be prokaryotic cells or eukaryotic cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: yeast cells, mammalian cells, bacterial cells, and insect cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: human cells, mouse cells, rat cells, bovine cells, porcine cells, hamster cells, and monkey cells. In a particular embodiment, the cells that have been transfected with an expression vector that directs the expression of the melanocortin receptor are selected from the group consisting of: L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 cells (ATCC CRL 1573), Raji cells (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-

C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). In a particular embodiment, the cells are not *Xenopus* melanophore cells.

In a particular embodiment, the melanocyte stimulating hormone is selected from the group consisting of:  $\alpha$ -melanocyte stimulating hormone,  $\beta$ -melanocyte stimulating hormone, and  $\gamma$ -melanocyte stimulating hormone.

In a particular embodiment, the ART polypeptide has an amino acid sequence selected from the group consisting of: SEQ.ID.NOs.1-19 and 20.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the conditions under which the method is practiced are conditions that are typically used in the art for the study of protein-protein interactions: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C.

In particular embodiments of the above-described method, the chosen concentration of the melanocyte stimulating hormone is from 0.05 nM to 2.0 nM, preferably from 0.1 nM to 1.0 nM, and more preferably from 0.2 nM to 0.5 nM.

In particular embodiments of the above-described method, the chosen concentration of the ART polypeptide is from  $10^{-12}$  M to  $10^{-7}$  M.

In particular embodiments of the above-described method, the method is practiced *in vitro* and the melanocyte stimulating hormone is labeled, *e.g.*, enzymatically, radioactively, or the like, and the amount of binding of the melanocyte stimulating hormone to the melanocortin receptor is measured by determining the amount of label bound to the cells containing the melanocortin receptor.

Step (b) of the above-described method can be modified in that, rather than exposing intact cells to the melanocyte stimulating hormone, the ART polypeptide, or the substance, membranes can be prepared from the cells and the membranes can be exposed to the melanocyte stimulating hormone, the ART polypeptide, or the substance. Such a modification utilizing membranes rather than intact cells in methods similar to that described above, although directed to the binding interactions of other ligands and receptors, is well known in the art and is described in, *e.g.*, Hess *et al*, 1992, Biochem. Biophys. Res. Comm. 184:260-268.

As a further modification of the above-described method, RNA encoding the melanocortin receptor can be prepared as, *e.g.*, by *in vitro* transcription

using a plasmid containing nucleotide sequences encoding the melanocortin receptor under the control of a bacteriophage T7 promoter, and the RNA can be microinjected into *Xenopus* oocytes in order to cause the expression of the melanocortin receptor in the oocytes. These oocytes then take the place of the cells in the above described method.

Once a substance has been identified as an inhibitor of the binding of the ART polypeptide to the melanocortin receptor, that substance can be tested to determine whether it is also an agonist of the melanocortin receptor. Such testing would involve exposing cells that express the melanocortin receptor to the substance, in the absence of the melanocyte stimulating hormone and the ART protein or ART polypeptides, and determining whether the melanocortin receptor is thereby activated by the substance. In this way, an inhibitor of the effect of ART protein on MC3R or MC4R can be identified that has no, or little, MC3R or MC4R agonist activity, but that relieves the inhibition of MC3R or MC4R receptor activity produced by ART protein. In a similar manner, it can be determined whether the substance is an antagonist of the melanocortin receptor.

Compared to full-length ART protein, the ART polypeptides of the present invention are smaller, and therefore easier to produce and less likely to be degraded. With respect to such embodiments of the invention as, *e.g.*, c-ART-b, the non-ART protein amino acid sequences added to the C-terminus of the ART sequences do not impair binding or functional activity, and allow <sup>32</sup>P or <sup>33</sup>P labeling without the need to label the ART sequence. Fusion polypeptides such as, *e.g.*, ART-AP or ART-luc, allow the use of non-radioactive methods to detect ART polypeptides in binding assays.

That the ART polypeptides of the present invention having amino acid sequences from non-ART proteins at their C-terminus are functional is surprising. The C-terminus of ART protein is homologous to the C-terminus of the agouti protein, both the ART protein and the agouti protein having a characteristic pattern of cysteine residues in this region. A similar pattern of cysteine residues has been found in certain ion channel blockers from spider and snail toxins. This pattern of cysteines has been proposed to result in the formation of specific disulfide bridges that constrain the toxins into a characteristic three-dimensional structure that is responsible for the toxins' biological activity (Kim *et al.*, 1995, J. Mol. Biol. 250:659-671; hereinafter "Kim"). While the extreme C-terminal amino acids of the toxin

studied by Kim were not part of this three-dimensional structure, these extreme C-terminal amino acids were nevertheless "crucially important," since altering them resulted in a loss of activity. See page 665, right column of Kim: "These results suggest that the C-terminal segment of  $\omega$ -AGA-IVA is crucially important for its blocking action on the P-type calcium channel expressed in rat cerebellar Purkinje cells." Thus, one would have expected that altering the C-terminus of the ART protein, *e.g.*, by linking it to sequences from a non-ART protein, would have resulted in an ART fusion polypeptide which would lack the activity of the full-length ART protein, or at least show substantially less activity. The present invention demonstrates that this is not so.

The following non-limiting examples are presented to better illustrate the invention.

#### EXAMPLE 1

##### Production of a construct expressing c-ART-b

The expression plasmid for c-ART-b was constructed by modifying the ART expression plasmid which was generated by inserting the ART cDNA into the EcoRI and BamHI sites of pcDNA3.1-Myc-His-A (Fong *et al.*, 1997, Biochem. Biophys. Res. Comm. 237:629-631; hereinafter "Fong"). The c-ART-b sequence differs from that of the recombinant ART as described by Fong in that residues 27-75 were deleted from ART. To accomplish that, a first PCR was carried out using the ART expression plasmid as template and two oligos (GGGCTCGGCGGTCCTGCAGGGCCAAGCCCATCTGGGC (SEQ.ID.NO.:21); and the T7 primer TAATACGACTCACTATAGGG (SEQ.ID.NO.:22)) to amplify the DNA fragment encoding ART residues 1-26 followed immediately by residues 76-81. A second PCR was carried out using the ART expression plasmid as template and two other oligos (CTGCAGGACCGCGAGCCC (SEQ.ID.NO.:23); and the pcDNA3.1A primer GTCGACGGCGCTATTTCAG (SEQ.ID.NO.:24)) to amplify the DNA fragment encoding ART residues 76-132. A third PCR was then carried out using the first and the second PCR products as template and two oligos (the T7 primer and the pcDNA3.1A primer) to amplify the c-ART-b cDNA. The final PCR product

was cleaved by the restriction enzymes EcoRI and BamHI, and ligated to the pcDNA3.1-Myc-His-A vector similarly cleaved by EcoRI and BamHI. The thrombin site sequence was based on the thrombin site in pET-34b (Novagen, Milwaukee, WI). The Myc epitope sequence and hexahistidine sequence were contained within the

5 pcDNA3.1-Myc-His-A vector (Invitrogen, Carlsbad, CA).

## EXAMPLE 2

### Expression of c-ART-b

COS-7 cells in T-175 flasks were transiently transfected with the c-ART-b expression plasmid (24 µg) by lipofectamine (Gibco), and grown in Opti-mem media (Gibco) supplemented with 1% fetal bovine serum. Two days after transfection, culture media were collected, centrifuged to remove residual cells, concentrated about 100-fold in Centriprep-3 (Amicon) and stored at 4°C in the presence of 2.5 mM EGTA, 4 mg/ml leupeptin, and 0.01 mM phosphoramidon. After

10 determination of the concentration of c-ART-b, NaN<sub>3</sub> was added to 0.02%.

Determination of the concentration of c-ART-b, which contains the Myc sequence, was based on an ELISA standard curve. Briefly, the microtiter plate was coated with 0.2 µg of a Myc peptide (human c-Myc 408-439) overnight, washed, blocked, and followed by incubation with anti-Myc mAb-HRP conjugates (Invitrogen) in the

15 presence of varying concentrations of the free Myc peptide for 2 hours. The bound mAb-HRP was detected using a colorimetric substrate tetramethylbenzidine (BioRad). For c-ART-b concentration determination, a c-ART-b sample replaced the free Myc peptide.

20

## 25 EXAMPLE 3

### Binding of c-ART-b to MC3R and MC4R

Binding assays were done in the same manner as described in Fong *et al.*, 1997, Biochem. Biophys. Res. Comm. 237:629-631. Binding assays were carried out using membranes prepared from L cells or CHO cells stably expressing human

30 MC3R, MC4R or MC5R. The binding assay mixture contained 0.2 nM of <sup>125</sup>I-

[Tyr<sup>2</sup>][Nle<sup>4</sup>, D-Phe<sup>7</sup>]  $\alpha$ -melanocyte stimulating hormone (<sup>125</sup>I -NDP- $\alpha$ -MSH), varying concentrations of c-ART-b or full-length ART protein, and an appropriate amount of membranes so that the total bound radioligand was less than 10% of the added radioligand. The above mixture in binding buffer (50 mM Tris, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM KCl, pH 7.2) was incubated at room temperature for 2 hours, followed by filtration through GFC paper. The bound ligand was quantitated in a  $\gamma$  counter. IC<sub>50</sub> values were calculated as previously described (Fong *et al.*, 1996, Mol. Pharmacol. 50:1605-1611). The results are shown in Figure 2. From Figure 2 it can be seen that c-ART-b inhibits the binding of <sup>125</sup>I-NDP- $\alpha$ -MSH to MC3R.

A similar experiment was done to determine whether c-ART-b inhibits the binding of <sup>125</sup>I-labeled NDP- $\alpha$ -MSH to MC4R. The results are shown in Figure 3. From Figure 3 it can be seen that c-ART-b inhibits the binding of <sup>125</sup>I-NDP- $\alpha$ -MSH to hMC4R.

Similar experiments were performed with full-length human ART protein. Similar experiments were also performed with full-length ART protein and with c-ART-b for the melanocortin-5 receptor (MC5R). From these experiments, from the results shown in Figures 2 and 3, and from similar experiments, the following IC<sub>50</sub> values for the inhibition of <sup>125</sup>I-labeled NDP- $\alpha$ -MSH to MC3R, MC4R, and MC5R by full-length ART protein and by c-ART-b can be determined.

Table 1

	hMC3R	hMC4R	hMC5R
full length ART	1.0 $\pm$ 0.4 (4)	0.5 $\pm$ 0.1 (3)	>40
c-ART-b	1.9 $\pm$ 1.0 (2)	1.4 $\pm$ 0.1 (2)	not done

The IC<sub>50</sub> values shown in Table 1 are given in nM. The numbers in parentheses represent the number of experiments run. The results shown in Table 1 indicate that, surprisingly, c-ART-b, although missing a significant amount of sequence from the N-terminus of the ART protein, is essentially functionally equivalent to full length ART protein. In addition, c-ART-b is functional despite having a significant amount of non-ART sequences at its C-terminus (a thrombin site, a myc epitope, and a hexahistidine tag).

## EXAMPLE 4

Functional assay for the binding of c-ART-b to MC3R and MC4R

- The ability of c-ART-b to inhibit the production of cAMP by  $\alpha$ -melanocyte stimulating hormone acting through MC3R or MC4R can be demonstrated by preincubating L Cells stably expressing human MC3R or MC4R with c-ART-b for 10 minutes, followed by incubation with 20 nM  $\alpha$ -melanocyte stimulating hormone for 45 minutes. The incubation buffer also contains Earle's balanced salt solution, 10 mM HEPES, 5 mM MgCl<sub>2</sub>, 1 mg/ml BSA and 0.5 mM IBMX. -Following the incubation, cells are lysed by boiling for 4 minutes.
- 5
- 10 Intracellular cAMP concentration is measured by RIA (Huang *et al.*, 1997, J. Receptor Signal Transduc. Res. 17:599-607) using anti-cAMP antibody and <sup>125</sup>I-cAMP as modified in the scintillation proximity assay format (Amersham).

- The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.
- 15

- Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.
- 20